

Amendments to the Specification:

Please replace the paragraphs beginning at page 65, line 17, through page 66, line 26 with the following rewritten paragraphs:

3. Preparation of the Soluble Fraction

For gel analysis, about 500 μ l of acetone was added to 125 μ l of each soluble fraction (about half of the volume generated) to precipitate the protein. The samples were left at room temperature for about 15 minutes followed by centrifugation for 5 minutes in a microfuge. The protein precipitates were then each resuspended in ~~25 μ l of dH₂O + 25 μ l~~ 25 μ l of dH₂O + 25 μ l of 2X Sample Buffer. The samples were then heated for about 3-5 minutes at about 90°C, vortexed well and allowed to cool to room temperature. A final 5 minute centrifugation was then done and the supernatants were transferred to clean tubes.

4. Preparation of the Insoluble Fraction

The cell pellets, or insoluble fractions, generated following sonication of the cells, were each resuspended in ~~100 μ l~~ 100 μ l of TE (10 mM Tris pH 7.6, 1 mM EDTA) + ~~20 μ l~~ 20 μ l 10% SDS and vortexed well. The samples were then heated at 90°C for about 3 minutes and vortexed hard again. After cooling to room temperature, about 500 ~~μ l~~ μ l of acetone was added to the samples to precipitate the protein and they were left at room temperature for about 15 minutes followed by centrifugation in a microfuge for 5 minutes. The pellets were then resuspended in ~~50 μ l of dH₂O + 50 μ l~~ 50 μ l of dH₂O + 50 μ l of 2X sample buffer. The samples were then heated for about 3-5 minutes at about 90°C, vortexed well and allowed to cool to room temperature. A final 5 minute centrifugation was then done and the supernatants, designated SDS Soluble, were transferred to clean tubes.

The pellets from the final centrifugation of the SDS Soluble Fraction were further processed. These pellets were each resuspended in ~~40 μ l dH₂O + 10 μ l~~ 40 μ l of dH₂O + 10 μ l 1M DTT + ~~50 μ l~~ 50 μ l 2X sample buffer. The samples were then heated for about 3-5 minutes at about 90°C, vortexed well, allowed to cool to room temperature and centrifuged for 5 minutes in a microfuge. The supernatants, designated SDS/DTT soluble, were transferred to clean tubes.

B. Immunoblot Analysis

Following preparation, 5 ~~μl~~ μl of each sample (soluble, SDS soluble and SDS/DTT soluble) was mixed with 1 ~~μl~~ μl of 1M DTT and loaded onto a 10 well, ~~4.0mm~~ 1.0 mm NOVEX manufactured 12% Tris-Glycine SDS-PAGE and electrophoresed at about 120 volts for 1.5 - 2 hours. The resulting gels were then used for immunoblots.

The SDS-PAGE gels were electroblotted onto a nitrocellulose membrane (NOVEX). The membrane was then blocked using a solution of 1X NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.05% ~~Triton~~ Triton[®] X-100) + 0.5% gelatin for approximately 30 min. - 1 hour rocking at room temperature. Following the blocking step, the membrane was placed in a solution of 1X NET + 0.5% gelatin + anti-Fab antibody (peroxidase-conjugated goat IgG fraction to human IgG Fab; CAPPEL #55223). The anti-Fab antibody dilution ranged from 1:50,000 to 1:1,000,000 depending on the lot of antibody. The membrane was left in the antibody solution overnight at room temperature with rocking. The next morning, the membrane was washed a minimum of 3 x 10 minutes in 1X NET + 0.5% gelatin and then 1 x 15 minutes in TBS (20 mM Tris pH 7.5, 500 mM NaCl). The protein bands bound by the anti-Fab antibody were visualized by using Amersham Pharmacia Biotech ECL detection and exposing the membrane to ~~X-Ray~~ x-ray film.

Please replace the paragraph beginning at page 68, line 10 with the following rewritten paragraph:

The human variable domain subgroup sequences can be found in the Kabat database available at a number of locations on the internet, such as ~~http://www/kabatdatabase.com,~~ and have been described in Kabat et al., Sequences of proteins of immunological interest, Ed.5. Public Health Service, National Institutes of Health, Bethesda, MD, 1991. A consensus variable domain sequence for each subgroup was constructed by selecting the most frequently occurring amino acid for each position in the variable domain. The FR1 sequences corresponding to amino acids 1-25 for each subgroup are:

Please replace the paragraph beginning at page 69, line 8 with the following rewritten paragraph:

The expression cassette in each designed vector contains at least the following basic components: (1) *phoA* promoter for the control of transcription; (2) λ_0 terminator to end transcription; (3) the Shine-Dalgarno sequence from the ~~*E. coli*~~ *E. coli* *trp* or the heat stable enterotoxin II (STII) gene, or a combination of both, to facilitate translation. The basic components of bacterial expression cassettes are known in the art and have been described in, for example, Kikuchi et al., *Nucleic Acids Res.* 9(21):5671-5678 (1981) (for ~~phoA~~ *phoA* promoter); Scholtissek and Grosse, *Nucleic Acids Res.* 15:3185 (1987) (for λ_0 terminator); Yanofsky et al., *Nucleic Acids Res.* 9:6647-6668 (1981) (for *trp*); Picken et al., *Infect. Immun.* 42:269-275 (1983) for STII); and Chang et al., *Gene* 55:189-196 (1987) (for combination use of *trp* and STII Shine-Dalgarno sequence). Additionally, the STII signal sequence or silent codon variants thereof precedes the coding sequence for both light and heavy chains in all constructs described and directs the secretion of the protein into the periplasm. Picken et al., *Infect. Immun.* 42:269-275 (1983); Simmons and Yansura, *Nature Biotechnology* 14:629-634 (1996). In this design, the cistron unit for each chain is under the control of its own *PhoA* promoter and is followed by a λ_0 terminator. Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques and or other molecular cloning techniques known in the art. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

Please replace the paragraphs beginning at page 71, line 1 through page 72, line 25 with the following rewritten paragraphs:

1. Shake Flask Inductions

Plasmids prepared as described in Section A were transformed into strain 33D3 (W3110 kan^R Δ fhuA (Δ tonA) ptr3 lacIq lacL8 ompT Δ (nmpr-fepE) deg P). Transformants were inoculated into 5 ml Luria-Bertani medium plus carbenicillin (50 ug/ml) and grown overnight at 30° C. Each culture was then diluted (1:100) into C.R.A.P. phosphate-limiting media (3.57g (NH₄)₂SO₄, 0.71g NaCitrate-2H₂O, 1.07g KCl, 5.36g Yeast Extract (certified), 5.36g HycaseSF-Sheffield, pH adjusted with KOH to 7.3, qs to 872 ml with SQ H₂O and autoclaved; cooled to 55°C and supplemented with 110 ml 1M MOPS pH 7.3, 11 ml 50% glucose, 7 ml 1 M MgSO₄ plus carbenicillin (50 ug/ml) and grown for about 24 hours at 30°C on a culture wheel.

2. Preparation of Samples for SDS-PAGE

Non-reduced whole cell lysates from induced cultures were prepared as follows: (1) 1 OD₆₀₀ -ml pellets were centrifuged in a microfuge tube; (2) each pellet was resuspended in 90 μ l TE (10mM Tris pH 7.6, 1mM EDTA); (3) 10 μ l of 100 mM iodoacetic acid (Sigma I-2512) was added to each sample to block any free cysteines and prevent disulfide shuffling; (4) 20 μ l of 10% SDS was added to each sample. The samples were vortexed, heated to about 90°C for about 3 minutes and then vortexed again. After the samples had cooled to room temperature, about 750-1000 μ l acetone was added to precipitate the protein. The samples were vortexed and left at room temperature for about 15 minutes. Following centrifugation for 5 minutes in a microcentrifuge, the supernatant of each sample was aspirated off and each protein pellet was resuspended in 50 μ l dH₂O + 50 μ l 2X NOVEX sample buffer. The samples were then heated for about 3-5 minutes at about 90°C, vortexed well and allowed to cool to room temperature. A final 5 minute centrifugation was then done and the supernatants were transferred to clean tubes.

Reduced samples were prepared by following steps similar to the steps described above for non-reduced samples, except that 10 μ l of 1M DTT was added to the cell resuspension solution in Step (2) and the addition of IAA was omitted in Step (3). Reducing agent was also added to a concentration of 100 mM when the protein precipitate was resuspended in 2X NOVEX sample buffer + dH₂O.

3. Immunoblot Analysis

Following preparation, 5-10 ~~μl~~ μl of each sample was loaded onto a 10 well, 1.0 mm NOVEX manufactured 12% Tris-Glycine SDS-PAGE and electrophoresed at about 120 volts for 1.5 - 2 hours. The resulting gels were then used for immunoblots.

The SDS-PAGE gels were electroblotted onto a nitrocellulose membrane (NOVEX). The membrane was then blocked using a solution of 1X NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.05% ~~Triton~~ Triton[®] X-100) + 0.5% gelatin for approximately 30 min. - 1 hour rocking at room temperature. Following the blocking step, the membrane was placed in a solution of 1X NET + 0.5% gelatin + anti-Fab antibody (peroxidase-conjugated goat IgG fraction to human IgG Fab; CAPPEL #55223). The anti-Fab antibody dilution ranged from 1:50,000 to 1:1,000,000 depending on the lot of antibody. The membrane was left in the antibody solution overnight at room temperature with rocking. The next morning, the membrane was washed a minimum of 3 x 10 minutes in 1X NET + 0.5% gelatin and then 1 x 15 minutes in TBS (20 mM Tris pH 7.5, 500 mM NaCl). The protein bands bound by the anti-Fab antibody were visualized by using Amersham Pharmacia Biotech ECL detection and exposing the membrane to X-Ray film.

The relative amounts of the completely assembled product in bands detected on the immunoblot were measured using scanning densitometry. The net intensity (in pixels) of each full-length completely assembled antibody band (i.e. the top band on the immunoblots) was determined using the following tools: Kodak Digital Science Image Station 440CF, Software: Kodak Digital Science 1D Image Analysis Software (v. 3.0.2), System: Microsoft ~~Windows~~ Windows[®] 95. The net intensity of each of the full-length completely assembled antibody bands for the variant antibodies with framework changes were then divided by the net intensity of the unmodified antibody to provide a value for the yield. The unmodified or control antibody was assigned a value of 1.

Please replace the paragraph beginning at page 73, line 17 with the following rewritten paragraph:

Following inoculation of the fermentor with the cell-containing medium from the shake flask, the culture was grown in the fermentor to high cell densities using a computer-based

algorithm to feed a concentrated glucose solution to the fermentor. Ammonium hydroxide (58% solution) and sulfuric acid (24% solution) were also fed to the fermentor as needed to control pH. Additions of L-61 (an antifoam – others can be used) were also used in some cases to control foaming. When the culture reached a cell density of approximately 40 ~~OD₅₅₀~~ OD₅₅₀, an additional 100 ml of 1M magnesium sulfate was added to the fermentor. Additionally, a concentrated salt feed (12.5 g ammonium sulfate, 32.5 g potassium phosphate dibasic, 16.25 g sodium phosphate monobasic dihydrate, 2.5 g sodium citrate dihydrate, 18.75 g potassium phosphate monobasic, 10 ml of 2.7% ferric chloride and 10 ml of trace elements in a final volume of 1250 ml) was added to the fermentor and started at a rate of 2.5 ml/min when the culture reached approximately 20 ~~OD₅₅₀~~ OD₅₅₀ and continued until approximately 1250 ml were added to the fermentation. Fermentations were typically continued for 70-80 hours. During the fermentation, once the dissolved oxygen set point for the fermentation was reached, the concentrated glucose solution was fed based on the dissolved oxygen probe signal in order to control the dissolved oxygen concentration at the set point. Consequently, in this control scheme, manipulations of fermentor operating parameters such as the agitation rate or back pressure which affect the oxygen transfer capacity in the fermentation correspondingly also manipulated the oxygen uptake rate or metabolic rate of the cells. A mass spectrometer was used to monitor the composition of the off-gas from the fermentations and enable the calculation of the oxygen uptake and carbon dioxide evolution rates in the fermentations.

Please replace the paragraphs beginning at page 75, line 11 through page 75, line 24 with the following rewritten paragraphs:

The VEGF-binding affinities of full length antibodies produced in bacterial cells were calculated from association and disassociation rate constants measured using a BIAcore™-2000 surface plasmon resonance system (BIAcore, Inc., Piscataway, NJ) as described in Chen et al, (1999) ~~J. Mol. Bio.~~ (1999) J. Mol. Biol. 293:865-881). A biosensor chip was activated for covalent coupling of VEGF using N-ethyl-N'-(3-dimethylaminopropyl)-carbo-dimide hydrochloride (EDC) and N-hydroxysuccinimid (NHS) according to the supplier's (BIAcore, Inc., Piscataway, NJ) instructions. VEGF (109) or VEGF (165) was buffer-exchanged into 20 mM sodium acetate, pH 4.8 and diluted to approximately 50 µl/minute to achieve approximately

700-1400 response units (RU) of coupled protein. A solution of 1 M ethanolamine was injected as a blocking agent.

For kinetics measurements, twofold serial dilutions of full length antibodies were injected in PBS/~~Tween~~ Tween[®] buffer (0.05% ~~Tween-20~~ Tween[®]-20 in phosphate-buffered saline) at 25°C or 37°C at a flow rate of 10 μ l/minute. Equilibrium dissociation constants, K_d values from SPR measurements were calculated as k_{off}/k_{on} .

Please replace the paragraphs beginning at page 80, line 9 through page 80, line 16 with the following rewritten paragraphs:

Some of the changes made during humanization to improve antibody affinity were made at positions that did not differ in amino acid sequence between the subgroup I and subgroup III sequence, such as at position 94 (Kabat numbering). This suggests that some additional modifications at positions other than those that differ ~~between~~ between the selected subgroup consensus sequence and the antibody variable domain sequence may be made in order improve binding affinity in the humanized antibody or antigen binding fragment.

When applying this method to humanized antibodies or antigen binding fragments, some of the FR region substitutions at the positions identified in accord with the methods of the invention may have already been made to improve antigen binding affinity, the improvement of the yield may be less than that would be expected if the changes to the subgroup III sequence had not already been made to the humanized antibody. Designing the anti-VEGF VNERK antibody with heavy chain FR regions from the human consensus subgroup I instead of subgroup III may have shortened the time to producing a humanized antibody or antigen ~~binding~~ binding fragment that can be produced in high yield in cell culture.

Please replace the paragraphs beginning at page 95, line 2 through page 95, line 18 with the following rewritten paragraphs:

Soluble fractions of each sample were prepared as follows: (1) a 5 O.D.₆₀₀ pellet of each sample was resuspended in 225 ~~μ l~~ μ l of 50 mM NaCl + 5 mM EDTA + 50 mM Tris pH 8 + 1 mg/ml lysozyme; (2) 25 ~~μ l~~ μ l of 100 mM IAA (Iodoacetic acid; Sigma I-2512) was then added; (3) the cell suspensions were vortexed and lysed by sonicating for 2 x 2 minutes at 50% pulse

(Sonics & Materials, Inc., Danbury, CT) (the samples were kept in an ice water bath during sonication to dissipate the heat generated during the process); (4) the samples were centrifuged for 5 minutes in a microfuge; (5) ~~100 μ l~~ 100 μ l of each supernatant (soluble fraction) was then acetone precipitated by adding approximately 500 ~~μ l~~ μ l of acetone to each sample and leaving the samples at RT approximately 15 minutes; (6) each precipitate was resuspended in ~~50 μ l of dH₂O + 50 μ l~~ 50 μ l of dH₂O + 50 μ l of 2X sample buffer; (7) the samples were heated at about ~~90~~ 90°C for 3-5 minutes, vortexed well, allowed to cool to RT; and, (8) the samples were centrifuged again for 5 minutes and the supernatants were transferred to clean eppendorf tubes.

The soluble fractions were then loaded (~~5-10 μ l~~ 5-10 μ l in each well) onto a 10 well, 1.0 mm NOVEX manufactured 12% Tris-Glycine SDS-PAGE and electrophoresed at about 120 volts for 1.5-2 hours. The resulting gels were either stained with Coomassie Blue or used for an immunoblot.